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MODULATING CHROMATIN BY TRANSCRIPTION AND NUCLEOSOME TURNOVER

A GENOME-WIDE STUDY IN FISSION YEAST

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Modulating chromatin by transcription and nucleosome turnover
A genome-wide study in fission yeast

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my late father and family

ABSTRACT

Eukaryotic DNA is wrapped around histone proteins to form a nucleosome, the basic structural unit of chromatin. Multiple levels of chromatin organization are required to compact DNA into chromosomes, to ensure an accurate cell division. The dynamic organization of chromatin modulates nuclear processes including transcription, replication and DNA repair, through altering the accessibility of DNA to regulatory proteins. Changes in chromatin structure are mediated by modification of histone proteins, remodeling of nucleosomes, incorporation of histone variants, histone turnover, noncoding RNAs and non-histone DNA-binding proteins. Chromatin is organized into active and repressed domains separated by chromatin boundaries. The establishment and maintenance of distinct chromatin domains has important implications in regulation of gene expression. In this thesis we have used fission yeast, *Schizosaccharomyces pombe* as a model organism to study the interplay between histone modifications, transcription and histone turnover in modulating chromatin. One of our main findings is that histone H2B is monoubiquitinated (H2Bub1) at centromeric chromatin. H2Bub1 dependent transcription of centromeric chromatin is required for establishment of active centromeric chromatin to ensure accurate chromosomal segregation during cell division. In this thesis we also showed that the Paf1/Leo1 heterodimer is involved in transcription dependent histone turnover to maintain active chromatin states. Loss of Paf1/Leo1 prevents histone turnover, which in turn leads to heterochromatin stabilization. In addition, by using Podbat, an in-house tool developed to visualize and analyze genome-wide data, we suggested a role for histone variant H2A.Z in DNA damage responses. We further proposed that H2A.Z is incorporated into nucleosomes in an Swr1-independent manner following genotoxic stress.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers:

I. **Sadeghi L**, Bonilla C, Strålfors A, Ekwall K, Svensson JP. Podbat: a novel genomic tool reveals Swr1-independent H2A.Z incorporation at gene coding sequences through epigenetic meta-analysis. *PLoS Comput Biol*. 2011 Aug; 7(8): e1002163

II. **Sadeghi L***, Siggens L*, Svensson JP†, Ekwall K†. Centromeric histone H2B monoubiquitination promotes noncoding transcription and chromatin integrity. *Nat Struct Mol Biol*. 2014 Mar; 21(3): 236-43

III. **Sadeghi L**, Prasad P, Ekwall K†, Cohen A†, Svensson JP†. The Paf1 complex factors Leo1 and Paf1 promote local histone turnover to modulate chromatin states in fission yeast. *EMBO Rep*. 2015 Oct 29. pii: e201541214

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CONTENTS

LIST OF ABBREVIATIONS

Ac	Acetylation
Ago1	Argonaute 1
ASF1	Anti-silencing function 1
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
Bp	base pair
Bre1	BREfeldin A sensitivity
CDK9	Cyclin-dependent kinase 9
CENP-A	Centromere protein A
CHD1	Chromo domain helicase DNA binding protein 1
ChIP	Chromatin immunoprecipitation
ChIP-chip	ChIP and DNA microarray
ChIP-exo	ChIP and lambda exonuclease digestion
ChIP-qPCR	ChIP and qPCR
ChIP-seq	ChIP and high-throughput sequencing
Chromo	Chromatin organization modifier
Clr	Cryptic loci regulator
CTD	C-terminal domain
Dcr1	Dicer 1
DNA	Deoxyribonucleic acid
DSB	DNA double-stranded break
FACT	Facilitates Chromatin Transcription
5FOA	5-Fluoroorotic Acid
H2Bub1	Monoubiquitinated H2B
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HIRA	Histone cell cycle regulation defective homolog A
HJURP	Holliday junction recognition protein
HR	Homologous recombination
Hrp1/3	Heterogeneous nuclear ribonucleoprotein 1/3

HP	Heterochromatin protein
IR	Inverted repeat
ISWI	Imitation Switch
K	Lysine
Kb	kilo base
Me	Methylation
me2	Dimethylation
me3	Trimethylation
MMS	Methyl methanesulfonate
4NQO	4-Nitroquinoline 1-oxide
ORF	Open reading frame
PEV	Position effect variegation
PTM	Posttranslational modification
Paf1	RNA polymerase associated factor 1
qPCR	Quantitative PCR
R	Arginine
Rad6	Radiation sensitivity protein 6
RITE	Recombination-induced tag exchange
RNA	Ribonucleic acid
RNAPII	RNA polymerase II
RNAPIII	RNA polymerase III
RNF20	Ring finger protein 20
RNF40	Ring finger protein 40
siRNA	Small interfering RNA
Scm3	Suppressor of chromosome missegregation protein 3
SNF	Sucrose non-fermentable
SWI	Switch
SWR1	SWI/SNF-related 1
TBZ	Thiabendazole
tRNA	Transfer RNA
TSS	Transcription start site

Ub	Ubiquitination
UV	Ultraviolet

1 INTRODUCTION

1.1 INTRODUCTION TO CHROMATIN STRUCTUR

1.1.1 Chromatin organization

The packaging and organization of eukaryotic genomic DNA to fit within the nucleus is a challenge for the cell. This is achieved by dynamic organization of DNA into chromosomes. In eukaryotic cells chromosomes are composed of DNA and protein complex organized into a structure called chromatin. The basic unit of chromatin structure is the nucleosome. The nucleosome consists of 147 base pair (bp) of DNA that is wrapped around an octamer of histone proteins (Figure 1) (Luger et al, 1997; Richmond & Davey, 2003). The core histone proteins are relatively small (11-15 KDa) and highly conserved among eukaryotic species. The histone octamer is composed of two copies of the four canonical histone proteins H2A, H2B, H3 and H4. Nucleosomal histones are organized as a single H3-H4 tetramer and two H2A-H2B dimers to form a histone octamer (Luger et al, 1997). The synthesis of the core histone proteins coincides with DNA replication during the S phase (Harris et al, 1991; Heintz et al, 1983). In each cell cycle, sufficient amounts of core histone proteins must be synthesized to package the newly replicated daughter DNA strands in nucleosomes and chromatin (Marzluff & Duronio, 2002).

The genome is organized into distinct chromatin domains based on different combination of regulatory proteins that affect the local composition of chromatin, which in turn influences the transcriptional activity of genes (Filion et al, 2010). In general chromatin is divided into two functionally and structurally distinguishable domains: euchromatin and heterochromatin reviewed in (Brown, 1966; Grewal & Jia, 2007). In 1928 Emil Heitz defined heterochromatin as chromosomal segments, which appeared extremely condensed at interphase (Heitz, 1928).

To facilitate access of nucleosomal DNA by regulatory proteins involved in transcription, replication and DNA damage responses, multiple mechanisms cooperate to alter chromatin structure. These mechanisms include ATP dependent chromatin remodeling, histone post-translational modifications (PTM), histone turnover and incorporation of histone variants. The N- and C-terminal region of histone proteins protrude out of the nucleosomes and is accessible for proteins involved in PTMs (Cosgrove et al, 2004). Moreover tail region mediates internucleosomal interactions within chromatin and is required for the folding of

nucleosome arrays into higher-order chromatin structure (Allan et al, 1982; Cavalli & Misteli, 2013). PTMs are essential for the maintenance of the epigenetic state of chromatin.

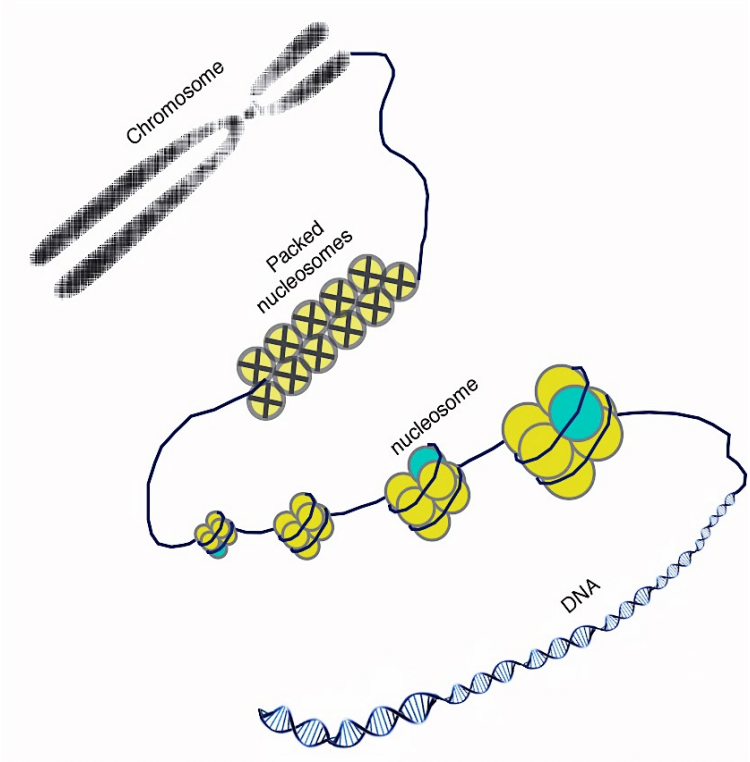

Figure 1. Eukaryotic DNA is packaged into chromosome. The DNA molecule is wrapped around histone octamer and compacted into chromatin. Nucleosomes contain either canonical histones (yellow) or histone variants (green). Nucleosomes are compacted and folded to form higher order chromatin structure. Compacted and folded chromatin forms chromosomes, which are visible by light microscopy during cell division.

The nucleosome positioning and occupancy throughout the genome governs the accessibility of the DNA to the regulatory proteins. ATP-dependent chromatin remodelers in coordination with chaperons mediate the nucleosome positioning in various ways.

1.1.2 Histone variants

In addition to canonical histone proteins, histone variants contribute to the diversity of chromatin structure and function. Histone variants are incorporated into the nucleosome at specific loci to alter chromatin structure and dynamics to carry out specific functions. (Talbert & Henikoff, 2010; Venkatesh & Workman, 2015).

Centromere specific histone H3 variant Cen-H3, known as CENP-A in mammals, Cse4 in *S. cerevisiae* and Cnp1 in *S. pombe* (Earnshaw & Rothfield, 1985; Stoler et al, 1995; Takahashi et al, 2000), is an essential protein that is localized to centromeres and plays an crucial function during cell division (Howman et al, 2000; Stoler et al, 1995). CENP-A creates a platform for kinetochore assembly at the centromere to ensure proper segregation of chromosomes during cell division (Black & Bassett, 2008). In each cell cycle CENP-A containing nucleosomes are divided between daughter strands of the replicating DNA thus each new centromere has only half of the previous number of CENP-A nucleosomes. Deposition of CENP-A nucleosomes occurs during early G1 in human cells (Dunleavy et al, 2011). A recent study by counting CENP-A^{Cnp1} molecules at single cell resolution in different phase of cell cycle showed that in *S. pombe* CENP-A^{Cnp1} deposition occurs during G2 (Lando et al, 2012). Scm3, a homolog of human HJURP, is a CENP-A^{Cnp1} chaperone involved in CENP-A deposition at centromeric chromatin in *S. pombe.* (Pidoux et al, 2009; Williams et al, 2009). Chromatin remodeling factor Hrp1, a homolog of Chd1, influences CENP-A^{Cnp1} loading to the centromere in *S. pombe* (Walfridsson et al, 2005). It has been suggested that RNAPII dependent transcription of promoters within the centromeric chromatin leads to $Chd1^{Hrp1}$ dependent exchange of H3 with CENP-A^{Cnp1} (Choi et al, 2011). This observation suggests that transcription of centromere core in *S. pombe* is required for CENP-A^{Cnp1} deposition. However it is not completely clear how transcription might influence CENP-A^{Cnp1} deposition.

H2A.Z, encoded by *htz1* in *S. cerevisiae* and *pht1* in *S. pombe* is the most conserved H2A variant among the eukaryotic species. H2A.Z is an essential gene for survival of *Drosophila* (van Daal & Elgin, 1992) and mice (Faast et al, 2001) but not for simple eukaryotes such as fission yeast (Carr et al, 1994) although *S. pombe* cells lacking *pht1* exhibit slower growth phenotype. Studies showed that H2A.Z is found at promoter region of lowly expressed genes in *S. pombe* and *S. cerevisiae* (Kim et al, 2009; Marques et al, 2010). Presence of H2A.Z within the promoter region anticorrelates with transcription levels in yeast (Millar et al, 2006; Zhang et al, 2005). Studies have indicated a connection between H2A.Z and various classes of inducible genes. Genes that are misregulated upon loss of H2A.Z in yeast tend to be involved only in specific environmental conditions (Halley et al, 2010; Millar et al, 2006). However, studies in animals have reported that H2A.Z shows a positive correlation with transcription level (Hardy et al, 2009). In *S. cerevisiae* the *htz1* deleted cells are sensitive to DNA damaging agents including MMS (Methyl methanesulfonate) and UV, implicating a role for H2A.Z in DNA damage repair (Mizuguchi et al, 2004). H2A.Z also contributes to

chromosome stability because H2A.Z deletion or knockdown leads to segregation defect and chromosome loss (Carr et al, 1994; Rangasamy et al, 2004). Moreover studies in budding yeast indicated that Htz1 acts as a barrier to prevent spread of silent heterochromatin. Htz1 was proposed to be involved in maintenance and establishment of the boundary between heterochromatin and euchromatin in *S. cerevisiae* (Meneghini et al, 2003). H2A.Z also plays a role in transcription elongation. In *S. cerevisiae htz1* deleted cells are sensitive to 6-Azuracil, transcription elongation inhibitor (Santisteban et al, 2011). H2A.Z can be acetylated on the four lysine residues within its N-terminal tail and acetylation is essential for its function (Kim et al, 2009; Millar et al, 2006)

Chromatin remodeling factor SWR1-C, a member of SWI/SNF family, is required for H2A.Z deposition into chromatin in *S. cerevisiae* (Krogan et al, 2003). H2A.Z physically interacts with Swr1, a subunit of SWR1-C in *S. cerevisiae* and deletion of *swr1* results in reduction of H2A.Z occupancy at promoter region of inducible genes (Mizuguchi et al, 2004). In *S. cerevisiae* Swr1 deposits H2A.Z-H2B dimers into the chromatin both *in vitro* and *in vivo*, however Swr1 does not remove H2A.Z from chromatin. A strong negative correlation between the genome-wide distribution of H2A.Z and *S. pombe* $Chd1^{Hrp1/Hrp3}$ remodeler has been observed, as H2A.Z was absent from $Chd1^{Hrp1/Hrp3}$ binding sites across the genome. This observation suggests that H2A.Z is deposited at promoters and is removed by the $Chd1^{Hrp1}$ remodeler when the gene is expressed (Buchanan et al, 2009).

1.1.3 Histone modifications

In addition to DNA compaction, histone proteins play essential roles in regulating gene expression and altering chromatin structure through post-translational modifications (Zentner & Henikoff, 2013). A large number of histone modifying enzymes regulate the PTMs of both non-bound histones as well as chromatin-bound histones. There are a variety of histone post-translational modifications that involve mostly lysine, arginine, threonine and serine amino acids (Kouzarides, 2007). The most studied modifications include acetylation (Sterner & Berger, 2000), methylation (Y. Zhang, 2006), phosphorylation (Nowak & Corces, 2004), ubiquitination (Shilatifard, 2006), sumoylation (Nathan et al, 2006), and ADP ribosylation (Hassa et al, 2006) of different residues in histone proteins. Histone modifications are carried out by a various number of enzymes including lysine methyltransferases (KMTs), lysine acetyltransferase (HAT) and E3 ubiquitin ligases. However, histone modifications are reversible and can be removed through activity of lysine demethylases (KDMs), histone

deacetylases (HDACs) and deubiquitinases reviewed in (Bannister & Kouzarides, 2011; Zentner & Henikoff, 2013). However there are some exceptions, for example a demethylase for H4K20me2/3 has not yet been found (Wang & Jia, 2009). It has been suggested that in *S. pombe* these modifications are removed from chromatin through histone turnover (Svensson et al, 2015) .

Histone PTMs influence the chromatin structure. For example histone acetylation neutralize the positive charge of histone proteins and weaken the interaction between histones and the DNA backbone. This leads to less compacted chromatin structure, which facilitates DNA accessibility by regulatory proteins (Campos & Reinberg, 2009; Hyland et al, 2005). Moreover, histone PTMs provide surfaces for the recruitment of regulatory proteins. A wide variety of chromatin associated factors have been shown to interact with modified histones. These interactions are mediated by specific domains within factors that allow simultaneous recognition of modifications. For example, heterochromatin protein 1 (HP1) recognizes H3K9me3, a mark associated with heterochromatin, via its chromodomain and this interaction is important for the maintenance of chromatin states (Lachner et al, 2001; Y. Zhang, 2006). A combination of different modifications can occur on various sites on histones for the tight control of chromatin structure. Histone cross talk between different modifications add additional levels of complexity and diversity to organization of chromatin (Fischle et al, 2003).

1.1.4 ATP dependent chromatin remodelers

Remodeling enzymes are able to translocate nucleosomes along the DNA, evict nucleosomes or exchange canonical histones with histone variants (Bartholomew, 2014; Hota & Bartholomew, 2011). Moreover remodeling enzymes generate and maintain TSS (transcription start sites) nucleosome depleted region (Alen et al, 2002; Walfridsson et al, 2007). Chromatin remodelers contain a conserved ATPase subunit and also different subunits/domains by which they can be recruited to chromatin. For example Switch (SWI)/SNF remodeling enzyme has a bromodomain that binds to acetylated histones and recruits remodeler to active chromatin (Zeng & Zhou, 2002). ATPase activity of remodeling enzymes is stimulated by binding to their substrates, which include nucleosomes or naked DNA. The major subfamilies of chromatin remodelers include (SWI)/SNF, Imitation Switch (ISWI), chromodomain Helicase DNA-binding (CHD), and Inositol requiring mutant 80 (INO80)/SWI/SNF-related 1 (SWR1) chromatin remodelers, classified based on sequence

homology of their conserved ATPase subunits and the presence of other domains (Bartholomew, 2014). Different subfamilies of remodeling enzymes have distinct modes of nucleosome remodeling around DNA.

1.2 HISTONE MONOUBIQUITINATION AND CHROMATIN DYNAMICS

1.2.1 Introduction to histone monoubiquitination

In mammals, both H2A and H2B are monoubiquitinated unlike in yeast, where monoubiquitination of H2A has not been reported (Swerdlow et al, 1990). H2A monoubiquitination site is mapped to lysine (K) 119 at the C-terminal region in human cells. H2A monoubiquitination is mediated by at least two different E3 ubiquitin ligases, Ring1B and 2A-HUB, both of which are associated with transcriptional silencing (Cao et al, 2005; Zhou et al, 2008).

H2B monoubiquitination occurs at the C-terminal region and is conserved from yeast to human (West & Bonner, 1980). The monoubiquitination of histone H2B occurs at K120 in human, K119 in fission yeast and K123 in budding yeast (Figure2) (Robzyk et al, 2000). The H2B monoubiquitination is mediated by the E3 ubiquitin ligase Bre1 in budding yeast and its homologs Brl1/Brl2 in fission yeast (Figure 3) and RNF20/RNF40 in mammalian cells (Kim et al, 2005; Tanny et al, 2007; Wood et al, 2003a). H2Bub1 is a highly dynamic process. Similar to other modifications, H2Bub1 is reversible. Ubiquitin can be removed by the action of deubiquitylating enzymes (DUBs). In yeast several H2B DUBs have been identified but the main ones are Ubp8 and Ubp10 in *S. cerevisiae* and Ubp8 and Ubp16 in *S. pombe* (Emre et al, 2005; Henry et al, 2003). In mammals USP22 has been reported to be involved in H2B deubiquitination (Zhang et al, 2008b). In addition to K120 H2B is also monoubiquitinated at K34 (H2BK34ub) in mammalian cells. The K34 is located within an extremely basic, eight residue patch on H2B N-terminal tail and is mediated by E3 ubiquitin ligase MSL1/2 (Wu et al, 2011).

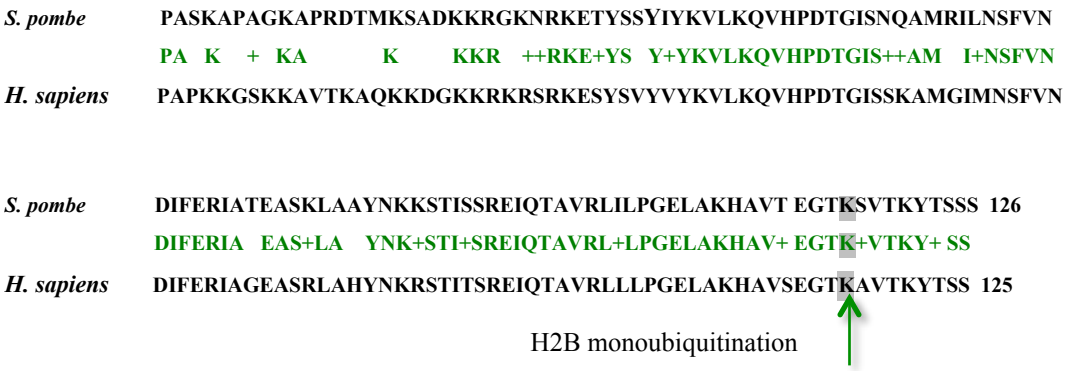

Figure 2. Amino acid sequence alignment of histone H2B in *H. sapiens* and *S. pombe.* Amino acid sequences are derived from the NCBI protein database. Arrow indicates H2B monoubiquitinated site in each spices.

1.2.2 H2B monoubiquitination and gene expression

Earlier work showed high frequency of monoubiquitinated H2B in transcriptionally active chromatin, suggesting a role for H2Bub1 in transcription (Nickel et al, 1989; Weake & Workman, 2008). Monoubiquitinated H2B is associated specifically with transcribed regions of actively transcribed genes (Minsky et al, 2008). In *S. cerevisiae* H2Bub1 contributes to the transcription of several inducible genes and the transcription of those genes is impaired in the absence of H2Bub1 (Kao et al, 2004). H2Bub1 generally correlates with RNAPII and transcription levels (Minsky et al, 2008; Sanso et al, 2012). In the absence of H2Bub1 in *S. pombe*, the RNAPII level within the transcribed regions is reduced, but only the transcription level of a sub set of genes is affected *(Sanso et al, 2012)*. In addition depletion of RNF20 in human cell lines has no effect on the levels of the majority of transcripts; as in H2Bub1 deficient *S. pombe* cells only transcription level of a subset of genes is altered (Shema et al, 2008).

Monoubiquitinated H2B serves as a mark to determine transcription elongation rate (Fuchs et al, 2014). H2Bub1 regulates transcription elongation by several mechanisms. *In vitro* transcription assays showed that H2Bub1 cooperates with the complexes Facilitates Chromatin Transcription (FACT) and Polymerase Associated Factor (PAF) to regulate transcription elongation (Pavri et al, 2006). FACT facilitates RNAPII passage through the chromatin by displacing of a single H2A-H2B dimer from the nucleosomes (Figure 3) (Belotserkovskaya, Oh et al. 2003). H2Bub1 increases the frequency of FACT mediated displacement of H2A-H2B dimer from the nucleosome and enhances transcription elongation (Belotserkovskaya et al, 2003; Pavri et al, 2006). In yeast by recruiting FACT, H2Bub1

mediates reassembly of nucleosomes at gene bodies in the wake of elongating RNAPII (Figure3) (Fleming et al, 2008). FACT has two main subunits in *S. pombe* including Spt16 and Pob3 (Lejeune et al, 2007). It has been shown Spt16 is essential for viability in yeast (Malone et al, 1991).

Phosphorylation of Ser2 (Ser2-P) at RNAPII C-terminal domain (CTD) is required for H2Bub1. Ser2 phosphorylation is mediated by cyclin-dependent kinase 9 (CDK9) in the transcribed region and is required for transcription elongation and mRNA processing (Pirngruber et al, 2009). In mammalian cells collide core domain WAC adaptor protein interacts with Ser2-P, mediated by CDK9, and at the same time recruits RNF20/RNF40 to chromatin for H2Bub1 (Zhang & Yu, 2011). In fission yeast *S. pombe,* spCdk9 is essential for viability. It phosphorylates both Ser5 and Ser2 at CTD of RNAPII (Pei et al, 2006). In *S. pombe* H2Bub1 facilitates Cdk9 recruitment and Ser2/Ser5-P of RNAPII CTD (Sanso et al, 2012).

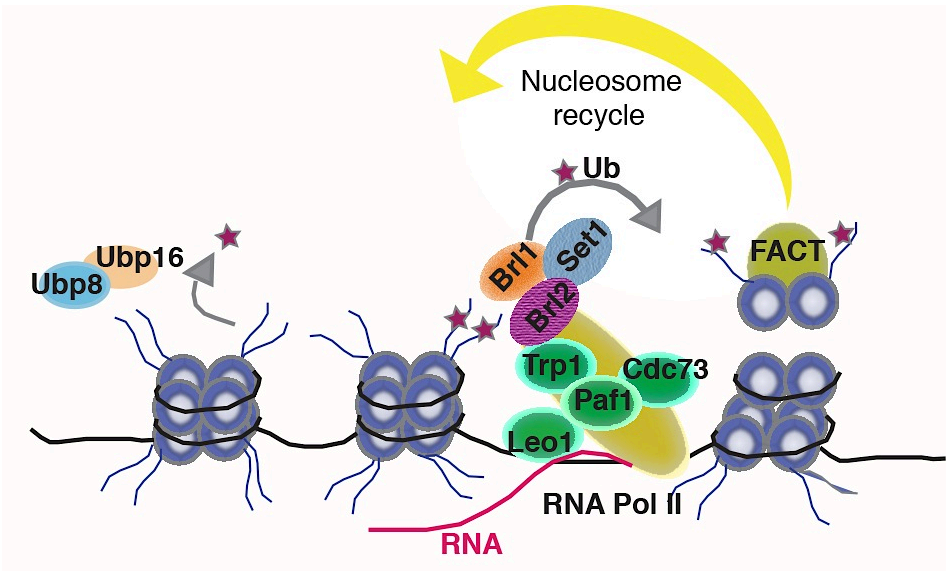

Figure 3. The role of H2Bub1 in transcription elongation. In *S. pombe* ubiquitin is transferred to histone H2B by Brl1/Brl2, which are associated with PAF1C. PAF1C facilitates H2Bub1 and transcription elongation. During transcription elongation, H2Bub1 stimulates H2A-H2B dimer removal by FACT and facilitates efficient RNAPII elongation through chromatin. FACT promotes recycling of the old histones during RNAPII passage. H2Bub1 is highly dynamic and must be removed through the activity of either Ubp8/Ubp16.

Furthermore, studies showed that PAF1 complex physically interacts with Bre1 in *S.*

cerevisiae. This interaction is required for recruitment of Bre1 to chromatin and linking the H2Bub1 and general transcription machinery (Kim & Roeder, 2009). PAF1 complex is required for the maintenance of H2Bub1 levels (Wood et al, 2003b). In *S. cerevisiae* Cdk9 homolog Bur1 Kinase is required for the efficient recruitment of the PAF1 complex and H2Bub1 (Zhou et al, 2009). PAF1 complex is a necessary cofactor for H2Bub1, and also plays an essential role in RNAPII transcription elongation and mRNA 3′ end processing (Tomson & Arndt, 2013).

Moreover, H2Bub1 facilitates transcription by recruiting regulatory proteins involved in histone methylation. In budding yeast, methylation of H3 at lysine 4 by Set1/COMPASS is dependent upon the H2Bub1 by Bre1 (Dover et al, 2002); revealing the presence of a trans-histone crosstalk between H2Bub1 and H3K4me. In the mutants lacking H2Bub1 (*rad6*Δ, *bre1*Δ or mutation at monoubiquitination-site *htb1*-K123R), the level of H3K4me was greatly reduced in *S. cerevisiae* (Sun & Allis, 2002).

1.2.3 H2B monoubiquitination and chromatin stability

H2Bub1 regulates global chromatin structure by stabilizing the nucleosomes and regulating chromatin dynamics. In *S. cerevisiae* H2Bub1 deficient cells showed less sensitivity to MNase digestion compared to WT. In contrast, *ubp8* deleted cells with elevated levels of H2Bub1 showed increased MNase sensitivity (Chandrasekharan et al, 2009). These results demonstrate that nucleosome stability is reduced or enhanced, when the H2Bub1 levels are abolished or increased, respectively. Furthermore in H2Bub1 deficient cells histones are more soluble at low salt concentration compared to WT, further suggesting that H2Bub1 affects nucleosome stability (Chandrasekharan et al, 2009). Additionally, in vitro experiment showed that the nucleosomes containing H2Bub1 exhibited slower rate of DNase I digestion compared to nucleosomes containing only H2B (Davies & Lindsey, 1994) .

Biochemical studies showed that the placement of 8.5 KDa ubiquitin moiety to the C-terminal of histone H2B alters chromatin structure, changes the conformation of chromatin and increases DNA accessibility for various chromatin modifying enzymes (Fierz et al, 2011). This suggests that, H2Bub1 stabilizes nucleosomes, provides a platform for the recruitment of regulatory proteins, and facilitates nucleosome disassembly and reassembly to permit the passage of RNAPII.

1.2.4 H2B monoubiquitination and other functions

In addition to regulating gene expression and chromatin stability, H2Bub1 has a role in DNA damage responses. The participation of H2Bub1 in DNA damage repair pathways was first implicated in *S. cerevisiae* as *bre1*Δ cells showed high sensitivity to ionizing radiation (IR) (Game et al, 2006). Later, studies revealed that RNF20/RNF40 play role in DNA damage responses in human cells. Both RNF20 and RNF40 were phosphorylated by ATM and recruited to the site of DNA damage (Moyal et al, 2011; Nakamura et al, 2011; Shiloh et al, 2011). The depletion of RNF20 by RNAi resulted in high sensitivity to IR and DNA damaging agents such as neocarzinostatin (NCS) and camptothecin (Chernikova et al, 2010; Moyal et al, 2011; Nakamura et al, 2011). Moreover RNF20 depleted cells are resistant to 4-NQO (4-nitroquinoline-1-oxide) a DNA damaging agent that induces base damage (Svensson et al, 2012). H2Bub1 is involved in DNA damage responses however the function of this modification in cellular responses to DNA damage is still not well understood. It is not clear whether H2Bub1 plays a role in the regulation of transcription at the site of DNA damage or has different functions.

H2Bub1 has diverse functions; it is required for methylation of kinetochore associated protein Dam1 in *S. cerevisiae* (Latham et al, 2011). Dam1 is required for proper kinetochore-microtubule attachment, suggesting a role for H2Bub1 in chromosome segregation. Moreover, recently H2Bub1 has been proposed to be involved in maintenance of chromatin boundary integrity. Loss of H2Bub1 resulted in impaired chromatin boundary function, which led to spreading of heterochromatin into adjacent euchromatic region (Ma et al, 2011). However, before studies presented in this thesis, it was not clear whether this effect was species specific and whether all chromatin domain boundaries were affected. Furthermore, it was not clear how H2Bub1 was associated with maintenance of distinct chromatin domains.

1.2.5 H2B monoubiquitination and cancer

During last few years, it has been shown that H2Bub1 is lost during carcinogenesis (Chernikova et al, 2012; Shema et al, 2008; Urasaki et al, 2012). Depletion of RNF20 in mammalian cells increases migration potential of cells and thereby facilitates metastasis, while depletion of RNF20 in breast cancer cells resulted in increased migration of tumor cells (Shema et al, 2008). RNF20 promoter is hypermethylated in breast cancer, which may serve to reduce RNF20 expression levels (Shema et al, 2008). RNF20 directly interacts with the p53 tumor suppressor protein and acts as a transcriptional coactivator (Kim et al, 2005)

suggesting that RNF20 functions as a tumor suppressor. Moreover deletion of mouse Bre1 leads to replication stress and formation of chromosomal aberrations, which can cause genomic instability (Chernikova et al, 2012). However additional studies will be required to specify the relationship between RNF20/RNF40 and chromosomal aberrations during cell division. It has been shown that RNAi mediated knockdown of the H2B ubiquitin ligase RNF40 decreased ERα induced gene transcription. ERα, estrogen receptor α, is a general hallmark of breast cancer. Proteasome inhibitor bortezomib treatment blocks estrogen induced growth and gene transcription in breast cancer cells through the reduction of H2Bub1 levels (Prenzel et al, 2011). Finally, direct studies on breast cancer samples demonstrated nearly complete loss of H2Bub1 in malignant tissues while adjacent nontransformed cells possessed substantial amount of this modification (Prenzel et al, 2011) Taken together, these observations suggest that loss of H2Bub1 correlates with cancer progression.

1.3 CHROMATIN DOMAINS

1.3.1 Introduction to chromatin domains

Studies have shown that histone modifying enzymes, chromatin remodeling complexes, histone turnover and DNA methylation are epigenetic components involved in organization of genome into distinct chromatin domains in eukaryotes (Jenuwein & Allis, 2001). Covalent modification of DNA is widely found in bacteria, plants, and mammalian cells and is associated with the epigenetic regulation of gene expression (Law & Jacobsen, 2010). Euchromatin is known to be more accessible for transcription as a result of less compaction whereas heterochromatin is highly compacted and enriched with repressive proteins (Grewal & Jia, 2007; Strålfors, 2011). Distinct chromatin domains contain specific profile of histone modifications that serve as epigenetic marks for chromatin structure and function. While methylated H3 on lysine 9 defines condensed heterochromatin domain, hyperacetylation of H3 (H3ac) or methylation at lysine 4 (H3K4me) is often associated with active genes (Cam et al, 2005; Santos-Rosa et al, 2002; Yan & Boyd, 2006). The repetitive DNA sequences are major component of heterochromatic regions. In fact heterochromatic silencing of repetitive DNA elements is important to protect the genomic integrity (Grewal & Jia, 2007; Miura et al, 2001). Heterochromatin can be subdivided into two classes, facultative and constitutive heterochromatin (Birchler et al, 2000; Brown, 1966). Constitutive heterochromatin consists of permanently silenced regions of the genome (Birchler et al, 2000). In *S. pombe* constitutive heterochromatin is localized across large chromosomal domains at centromeres, telomeres

and the mating type locus (Cam et al, 2005). Facultative heterochromatin includes genomic regions that are differentially expressed through development and differentiation. In *S. pombe* facultative heterochromatin is assembled over several meiotic genes including *mei4* and *ssm4* and also HOODs (heterochromatin islands including retrotransposons Tf2s), which are transcribed during meiotic differentiation (Cam et al, 2005; Horie et al, 1998; Zofall et al, 2012). In addition, LADs (lamina-associated domains) represent epigenetically silent domains enriched with H3K9me2/3 in human genome. Interaction between circadian genes and LADs at the nuclear periphery is required for the regulation of circadian transcriptional attenuation (Zhao et al, 2015). Thus LADs acts as heterochromatin domain involved in the regulation of rhythmic repression of circadian genes.

The centromere is part of the chromosome that is responsible for correct segregation during each cell division. In most organisms, the centromere locus is not determined by the DNA sequence whereas epigenetic marks are involved in centromere formation and maintenance (Sato et al, 2012). The centromere of *S. pombe* is arranged with a core centromeric region, which is flanked by the pericentric heterochromatin (Figure 4) (Clarke & Baum, 1990; Wood et al, 2002). The structural organization of the centromere in *S. pombe* is more complex than in *S. cerevisiae* and similar to the higher eukaryotes. The pericentric heterochromatin domain in *S. pombe* contains H3K9me2 and H3K9me3 (Nakayama et al, 2001; Yamada et al, 2005) and ensures proper segregation during cell division (Allshire et al, 1995; Ekwall et al, 1995). The core centromeric region where kinetochore is assembled contains Cen-H3 (Figure 4). In human cells, CENP-A nucleosomes are interspread with H3 nucleosome marked by H3K4me2 (Sullivan & Karpen, 2004). Centromeric chromatin in *S. pombe* is transcribed by RNAPII but transcripts are degraded by the exosome (Choi et al, 2011). Even though centromere DNA is transcribed in eukaryotes, the function of these transcripts is still not fully understood (Chen et al, 2015; Ferri et al, 2009). Moreover, the mechanism through which RNAPII is recruited to the centromeric chromatin has not been elucidated.

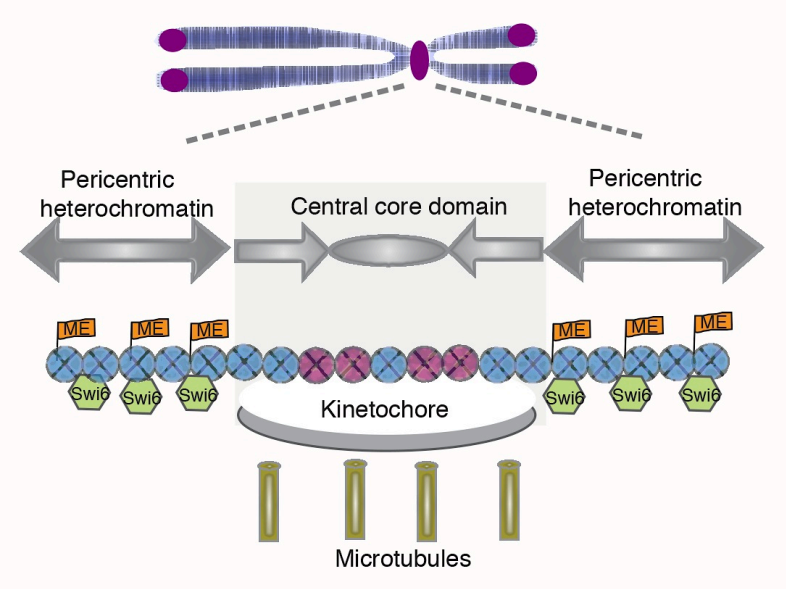

Figure 4. **Schematic view of *S. pombe* centromere**. In *S. pombe* chromosomes, constitutive heterochromatin is presented at centromere, telomeres and other silent regions. Centromeres are composed of pericentric heterochromatic regions occupied with H3K9me2 and Swi6 and central core domain that contains Cnp1 (pink) nucleosomes. The kinetochore is assembled at the central core domain and mediates microtubule attachment.

In addition to the telomere and pericentric region, another heterochromatic region in the *S. pombe* genome is the mating type locus. In homothallic strains, mating type region in addition to active *mat1* locus contains two transcriptionally inactive *mat2* and *mat3* loci, which are imbedded in heterochromatin. The silenced heterochromatic cen-H domain is located between *mat2* and *mat3* cassettes and is marked by H3K9me2 (Figure 5).

Methylation of histone H3 at lysine 9 (H3K9) by histone methyltransferases (HMT) Suv39h in human and Clr4 in *S. pombe* marks heterochromatin (Nakayama et al, 2001; Rea et al, 2000). Disruption of $Suv39h^{Clr4}$ HMT activities in *S. pombe* largely abolishes pericentric H3K9 methylation, indicating that this histone modification is an essential epigenetic mark for pericentric heterochromatin (Nakayama et al, 2001). Mammalian heterochromatin associated protein 1 (HP1), Swi6 in S. *pombe,* is a non-histone protein that acts in gene silencing. $HP1^{Swi6}$ interacts with H3K9me2/3 through its N-terminal chromodomain and this interaction is essential for the maintenance of heterochromatin (Bannister et al, 2001; Lachner et al, 2001). Moreover, $HP1^{Swi6}$ binds to the H3K9me mark and recruits additional nucleosome modifying enzymes that act on adjacent histones, resulting in the propagation of HP1 containing heterochromatin. Additionally the hypoacetylation of histone is required for heterochromatin maintenance (Casas-Delucchi et al, 2012). In *S. pombe* deacetylation of H3

on lysine 14 by the Clr3, a HDAC, is required for H3K9me2 by Clr4 and for Swi6 localization (Nakayama et al, 2001). Inhibition of HDACs in *S. pombe* resulted in loss of pericentric heterochromatin silencing, disrupted Swi6 localization and chromosome loss (Ekwall et al, 1997) . Histone deacetylation and methylation cooperate to establish a specific modification pattern for heterochromatin assembly.

The role of the RNAi machinery in heterochromatin establishment is well studied in *S. pombe* (Volpe et al, 2003). In *S. pombe* constitutive heterochromatic regions contain repetitive DNA elements. These elements are transcribed by RNAPII during S phase (Chen et al, 2008; Djupedal et al, 2005). These transcripts are converted to double-stranded RNA by RNA-dependent RNA polymerase (Rdp1) and processed by Dicer into small interfering RNA (siRNA). These siRNAs are loaded into Argonaute (Ago1) in the RITS (RNA-induced transcriptional silencing) complex (Verdel et al, 2004). The complex is associated with Clr4 to initiate H3K9me2 and further to recruit Swi6 (Bayne et al, 2010; Nakayama et al, 2001). The spread of heterochromatin from initiation site requires Swi6 (Zhang et al, 2008a). Moreover, the role of RNAi machinery in heterochromatin formation has been studied in other organisms including plants and *Drosophila* (Pal-Bhadra et al, 2004).

Furthermore, RNAi independent pathways are involved in heterochromatin formation. The heterochromatin assembly over meiotic genes requires transcription but occurs in an RNAi independent manner (Cam et al, 2005; Horie et al, 1998). The RNA surveillance factors Mmi1, Red1 and the exosome localize at meiotic genes and mediate heterochromatin assembly through Clr4 recruitment (Tashiro et al, 2013; Zofall et al, 2012). Additionally, in *S. pombe* transcription factors Atf1 and Pcr1 act together with an HDAC (Clr3) to establish heterochromatin formation at mating type locus independent of the RNAi machinery (Jia et al, 2004).

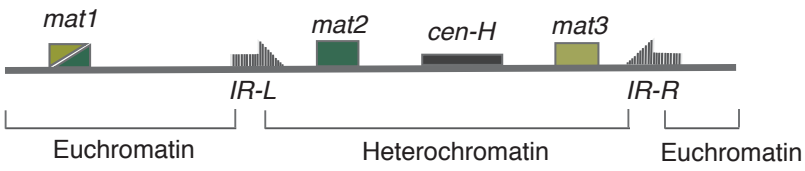

Figure 5. The mating-type region of *Schizosaccharomyces pombe.* The mating type locus is located on chromosome 2. *mat2* and *mat3* regions encode the information. The silenced heterochromatic region is surrounded by *IR* inverted repeat boundaries.

1.3.2 Chromatin domain boundaries

Boundaries define the border between heterochromatin and adjacent euchromatin. In the absence of boundaries, heterochromatin can spread into neighboring euchromatin and affect gene expression. Constitutive heterochromatin exerts a repressive effect on the expression of neighboring genes; this is the basis of position effect variegation (PEV). The PEV is the variation in the expression of a gene mediated by the random position of the gene in cells. Although PEV has been observed in many organisms from budding yeast to mammals (Dillon & Festenstein, 2002), it was originally described in *Drosophila* as a spotted red and white eye phenotype by Muller at 1930 (Muller, 1930). The mutant phenotype arises from a chromosomal rearrangement, which moves the gene from its wild type position to a position near to pericentric heterochromatin. The spreading of pericentric heterochromatin into a neighboring gene led to silencing of the white gene in some cells and cause a mosaic eye phenotype. In most cases, the specialized DNA elements known as insulators and their associated binding proteins are involved in establishment or maintenance of boundaries between distinct chromatin domains. The pioneering work on the existence of boundary elements and insulators came from the studies on *Drosophila*. Insulators and their interacting proteins form a looped domain that physically blocks the enhancer from reaching the promoter. The insulator is situated between the enhancer and the promoter and blocks the enhancer from driving gene transcription (Kellum & Schedl, 1992). The first vertebrate insulator was originally identified in the chicken β-globin locus. HS4 insulator at the 5′end of the locus can function as an insulator to prevent spread of heterochromatin into β-globin locus (Chung et al, 1993).

Transcription also plays a role in regulating boundary function through the recruitment of histone modifying activities and promoting nucleosome turnover. In *S. pombe* clusters of tRNA genes are found between centromeric chromatin and pericentric heterochromatin domains. These genes act as a barriers to limit heterochromatin spread (Partridge et al, 2000). Removal of these genes causes spreading of heterochromatin and silencing of marker genes inserted at the centromeric chromatin (Scott et al, 2006). The mechanism of tRNA boundary function is not completely understood, but the assembly of RNAPIII and the transcription factor TFIIIC is required (Noma et al, 2006; Scott et al, 2007). Moreover, histone demethylase Lsd1 is localized at the pericentric tRNA boundaries and demethylates H3K9me (Lan et al, 2007). Deletion of Lsd1 leads to expansion of H3K9me2 and H3K9me3, and Swi6 into the chromatin suggesting that Lsd1 functions to block heterochromatin spreading at the boundary region (Lan et al, 2007). In mammalian cells tRNA genes also function as

boundaries, suggesting an evolutionary conserved role for tRNA genes in preventing heterochromatin spread (Raab et al, 2012; Willoughby et al, 2000). In addition to tRNA genes, *IRC* elements surrounding cen1 and cen3 act as boundaries in *S. pombe*. The inverted repeats *IRC* elements are not associated with RNAPIII or the transcription factor TFIIIC (Noma et al, 2006). The bromodomain protein Bdf1is recruited to *IRC* boundary elements at pericentric region and it is required for H4K16 acetylation. Deficient H4K16 acetylation or *bdf1*Δ results in heterochromatin spreading across *IRC* boundaries (Wang et al, 2013). Additionally, Fun30 homolog Fft3 an ATP-dependent chromatin remodeling factor is localized at the centromeric boundary regions and protects centromeric domain from euchromatin formation (Stralfors et al, 2011). Another type of inverted repeats known as *IR* elements, flank the silent mating type cassettes and function as barrier (Figure 5). They contain several RNAIII B-Box sequences that recruit TFIIIC but not RNAPIII. It has been shown that TFIIIC mediates the clustering of chromosomal loci at the nuclear periphery (Noma et al, 2006).

In addition, a JmjC domain containing protein Epe1 is required for the boundary function of the pericentric *IRC* elements (Zofall & Grewal, 2006). Epe1 is recruited to heterochromatin by Swi6 and is highly enriched at *IRC* boundaries and negatively regulates heterochromatin spread (Trewick et al, 2007; Zofall & Grewal, 2006). Deletion of Epe1 results in heterochromatin spreading beyond its normal boundaries in *S. pombe* (Ragunathan et al, 2015; Zofall & Grewal, 2006). Although JmjC domain proteins are generally involved in histone demethylation, no such activity has been detected for Epe1 *in vitro* (Tsukada et al, 2006). Epe1 promotes histone exchange (Aygun et al, 2013) but it is not clear whether this function is direct or via an interactions with other proteins.

1.4 REPLICATION INDEPENDENT HISTONE TURNOVER

Histone exchange is a mechanism used by cell to keep chromatin dynamic, which involves ATP dependent removal of parts of the nucleosome or the entire nucleosome. This removal follows by the replacement with either newly synthesized histones or original component. Histone exchange influences the composition, structure and function of different genomic regions. Transcription and replication require chromatin disruption ahead of the RNA and DNA polymerase. Chromatin remodeling complexes contribute to both chromatin disassembly a head of the fork as well as reassembly of chromatin behind the fork.

Nucleosome turnover at promoters occurs more rapidly than in coding regions. Histone exchange at promoter region could be a way to add or remove certain histone modifications that plays a role during activation of transcription. Histone exchange rate over coding regions correlates with transcriptional activity (Dion et al, 2007; Rufiange et al, 2007). In *S. cerevisiae* study of histone turnover rate at G1 arrested cells showed that histone H3 exchange in gene body region strictly requires transcription, whereas a transcription independent H3 exchange occurs at promoters. This could indicate that nucleosomes within the promoter region are very dynamic and histone exchange in inactive promoters could poise them for transcription and facilitates rapid induction (Rufiange et al, 2007). Recently the recombination-induced tag exchange (RITE) method was used in *S. cerevisiae* to map differentially tagged old and new H3 during the cell cycle (Verzijlbergen et al, 2010). In *S. pombe* a recent study using RITE method showed that recycling of parental histones into the gene body region of actively transcribed genes result in a reduced histone turnover rates (Svensson et al, 2015). In yeast, FACT facilitates transcription through chromatin by removing one copy of the H2A-H2B dimer in a transcription dependent manner (Belotserkovskaya et al, 2003). Thus, FACT mediated histone recycles results in low incorporation of new histones into nucleosomes and preserves histone modifications. While histone eviction is necessary for RNAPII movement, histone reassembly is crucial for preventing cryptic transcription (Du & Briggs, 2010).

Histone H3.3 is a variant of histone H3 in higher eukaryotes. Histone H3.3 expressed throughout the cell cycle and deposited onto DNA in a replication independent manner (Ahmad & Henikoff, 2002). H3.3 has been linked to regions of high nucleosome turnover and has been associated with gene activation (Tagami et al, 2004).

Heterochromatin and euchromatin differ in the stability of nucleosomes. Heterochromatic regions are associated with more stable nucleosomes as a consequence of low nucleosome turnover (Aygun et al, 2013). An intrinsic mechanism mediates the eviction and reassembly of histones during RNAPII passage. Studies using newly synthesized tagged histones showed that some of the histones that reassembled into the chromatin during transcription are not the parental ones indicating the incorporation of new histones. However the incorporation of new histones into the chromatin challenge the stability of chromatin domains.

2 METHODOLOGY

2.1 SCHIZOSACCHAROMYCES POMBE

The fission yeast *Schizosaccharomyces pombe* commonly used, as a model organism is isolated from African beer. In 1950 *S. pombe* was introduced as a model organism for genetic and cell cycle studies by Leupold (Leupold, 1955). *S. pombe* has become a system to understand basic molecular and genetic processes in chromatin biology. *S. pombe* is easily manipulated genetically including deletion, overexpression and tagging. A collection of single gene mutant that covers 98.4% of the genes is available. *S. pombe* cells are rod shaped and have a genome size of 13.8-Mb distributed between 3 chromosomes. *S. pombe* is predicted to have a maximum of 4940 protein coding genes (Wood et al, 2002). It has been suggested that fission yeast diverged from budding yeast *S. cerevisiae* around 1,144-1,600 million years ago (Heckman et al, 2001). Therefore, although both *S. pombe* and *S. cerevisiae* are unicellular fungi, they also differ in many ways. *S. pombe* chromosome shares a number of important features with mammalian chromosome. Similar to mammalian cells, fission yeast has epigenetically defined regional centromeres that are surrounded by pericentromeric heterochromatin. Fission yeast has a complete RNAi machinery and has proven to be a powerful system to study the RNAi-directed heterochromatin formation (Aravind et al, 2000). However *S. pombe* is easily grown like *S. cerevisiae* with a short generation time of 2-4 hours (Figure 6).

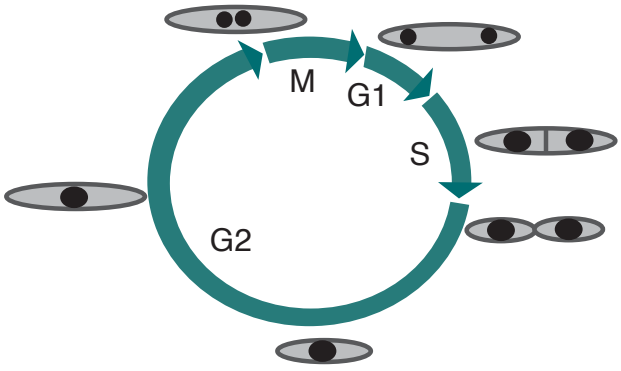

Figure 6. The vegetative life cycle of *Schizosaccharomyces pombe*. The mitotic fission yeast cell cycle, like other eukaryotes, is subdivided into G1, S, G2 and M. Cell growth predominantly occurs during G2, which constitutes about 70% of the cycle time, and unlike mammalian cells, cytokinesis occurs after S-phase

2.2 CHROMATIN IMMUNOPRECIPITATION

Chromatin immunoprecipitation (ChIP) is a method to study interaction between protein of interest and genomic DNA. The first ChIP assay was determined by Gilmour and Lis in 1984 to monitor the association of RNAPII and transcribed genes in *Escherichia coli* (Gilmour & Lis, 1984). ChIP coupled with genome-wide detection system are used to identify the location of proteins including transcription factors and histones at a genome-wide scale (Berman et al, 2009).

A method for using ChIP in *S. pombe* is described in (Durand-Dubief & Ekwall, 2009). Briefly, protein/DNA complexes are cross-linked *in vivo* using formaldehyde fixation (1% w/v final concentration), which is followed by glycine quenching. Then cells are lysed using glass beads and are fragmented randomly by sonication to approximately 400-500 bp. Then protein A-coated beads or magnetic beads and a specific antibody is used to select for fragments cross-linked to the protein of interest. After washing and elution from beads cross-links are reversed by heat treatment, and proteins are removed by digestion with proteinase K. Recovered DNA fragments can be quantified by PCR, microarray hybridization or sequencing (Haring et al, 2007; Pedrosa et al, 2009; Schones & Zhao, 2008). We used two types of control to normalize our ChIP data. First, a no-antibody control, which indicates the amount of background signal, generated during ChIP procedure. Second, unprecipitated chromatin extract (input) indicates the concentration of chromatin used in the ChIP reaction.

2.3 GENOME SCALE STUDIES

Chromatin immunoprecipitation followed by genomic tilling microarrays (ChIP-chip) or massively sequencing (ChIP-seq) are two of the widely used approaches to identify and characterize *in vivo* protein/DNA interactions. To study the histone modifications in genome-wide scale, we used both ChIP-chip and ChIP-seq methods in this thesis.

2.3.1 DNA Microarrays

The combination of ChIP and DNA microarrays was the beginning of a rapid progress in high-throughput studies. Microarray is made up of a collection of microscopic DNA spots attached to a solid surface. Briefly, samples including DNA fragments recovered from immunoprecipitation or cDNA (complementary DNA) are amplified and fluorescently labeled and hybridize to DNA microarray. The hybridization signal for each probe is detected by a detector (Gresham et al, 2008; Niemeyer & Blohm, 1999). The microarray Affymetrix

GeneChip S.pombe Tilling 1.0FR Array was used in this thesis. In this array probes are short and overlap slightly. This array covers the *S. pombe* entire genome with high resolution. The raw data produced by microarrays can be analyzed using Tiling Analysis Software (TAS) and visualization using the Integrated Genome Browser (IGB) or Podbat.

2.3.2 High throughput sequencing

In the past few years, the combination of ChIP with high throughput sequencing technology has become as an attractive alternative to ChIP-chip. Briefly, adaptors are ligated onto the ends of the ChIPed DNA and subjected to amplification. After size selection, the DNA is denatured into single strands and is then sequenced using the 454 sequencing or SoLid or Solex/Illumina technology. Sequencing is performed by identification of fluorescently labeled nucleotides that are incorporated during extension (Kircher & Kelso, 2010). Generation of a ChIP-seq profile requires different amount of sequencing data depending the size of genome. Increasing the depth of sequencing allows detection of sites with low level of enrichment over the genomic background. Sequencing data can be obtained from single or paired reads. In this thesis we used Solex/Illumina technology that is a high-throughput, short-read and massively parallel sequencing platform. Illumina provides HiSeq and MiSeq platforms generation 200 million and 15 million reads respectively. In this thesis, we generated data by Illumina's MiSeq and HiSeq platforms using single end reads. Even though both ChIP-chip and ChIP-seq identify regions when proteins are highly enriched, ChIP-seq has several advantages over ChIP-chip. ChIP-seq usually produces more distinct and narrow signal profile than ChIP-chip which shows higher signal to noise ratio. ChIP-seq produces profiles with higher resolution and more sensitivity and specificity over ChIP-chip. Moreover, many current ChIP-seq protocols work with a smaller amount of material compared to ChIP-chip (Ho et al, 2011). The cost of ChIP-seq is dropping rapidly and technique becoming more popular and user-friendly.

2.4 CHROMATIN IMMUNOPRECIPITATION AND EXONUCLEASE

The chromatin imunoprecipitation coupled with lambda exonuclease digestion (ChIP-exo) followed by high-throughput sequencing is a recently developed method to determine genomic location of proteins associated with genomic DNA at approximately nucleotide resolution (Rhee & Pugh, 2011; Svensson et al, 2015). Briefly the chromatin is immunoprecipitated with an antibody against a protein of interest. While protein/DNA cross-links are still on the beads, a 5′-3′ exonuclease digestion is employed to digest the 5′end of

the sonicated DNA fragments to the DNA/protein cross-linking point on one strand. However the 3′ end of sonicated DNA to the crosslinking point remains intact and is sufficiently long to uniquely identify in a genome when is sequenced. Then DNA/protein cross-links are eluted from bead and reversed and the DNA is extracted. To complete library construction the DNA is primer-extended and ligated with a second adaptor. After gel-purification and PCR amplification, the library is ready for high-throughput sequencing. The major advantage of ChIP-exo is high resolution over ChIP-chip and ChIP-seq. It also reduces signal to noise ratio by digesting contaminating DNA, therefore less sequencing depth is needed compared to ChIP-seq.

2.5 EPISTASIS ANALYSIS

The functional relationship between genes is important to understand both the structure and function of distinct genetic pathways. *S. pombe* and *S. cerevisiae* are used commonly for identification of new genes or the functional analysis of previously identified genes. Both can grow and divide as haploid. Both species have high rates of homologous recombination (HR) that facilitates genome editing. In this method a short DNA fragment with ends homologous to endogenous loci is introduced into the cells to incorporate into the DNA by HR and consequently edit the genome. When the gene function is abolished it is possible to identify its functions and interacting partners. However essential genes cannot be examined, because the mutated cells will be inviable. Conditional mutations can help to study the function of lethal genes including construction of temperature sensitive mutants. Epistasis is defined as interaction between different genes. In classical epistasis analysis, two genes are mutated in the same strain and the phenotype of the double mutant is compared with those of the corresponding single deleted mutants. Epistasis effects can be suppressive, additive or synergistic. Suppressive epistasis occurs when mutations have a negative influence on each other as a mutation corrects the phenotypic defects of another mutation without restoring its wild type sequence. Additive epistasis refers to combination of two different phenotypes resulting from double mutants, which indicates that the two mutations are in genes acting in distinct pathways. A synergistic interaction occurs where the combined effect of mutations is greater together than the sum of their individual effects. In harmful mutations, synergistic epistasis can result in lethality, where the combined effects of several harmful mutations are compounded by each other's presence leading to organism death. Genome-wide genetic interaction screens using mutations of genes have been performed in yeast in order to understand the association of genes network and map the genetic architecture (Kohli et al,

1977; Roguev et al, 2008; Zheng et al, 2010).

3 RESULTS AND CONCLUSIONS

3.1 PAPER I

The aim of this study was to introduce Podbat (Positioning database and analysis tool) an open source and user-friendly tool to visualize and analyze genome wide data. We used Podbat for reanalyzing all published genome-wide data for the histone variant H2A.Z in *S. pombe* as an example. By uploading H2A.Z enrichment data in WT and *swr1*Δ cells to Podbat, in agreement with previous studies, we observed that H2A.Z is present at the first nucleosome after transcription start site in Swr1 dependent manner.

Moreover, we found that H2A.Z is present at gene body region of genotoxic-induced genes in Swr1 independent manner. H2A.Z enrichment was independent of N-terminal acetylation because genes upregulated upon removal of H2A.Z N-terminal region followed the same pattern. In *S. cerevisiae* genes whose expression is induced by environmental stress are associated with H2A.Z at their coding region. In *S. cerevisiae htz1* deleted cells are sensitive to MMS. MMS is an alkylating agent that modifies DNA bases to cause DNA damage. By epistasis analysis it was shown that after MMS treatment correlation between Htz1 and Swr1 was lost (Bandyopadhyay et al, 2010). Similarly, after MMS treatment Htz1 became correlated with the DNA-damage checkpoint kinase Mec1 (a homolog of mammalian ATR/ATM). The double *mec1Δhtz1Δ* strain exhibited synthetic sensitivity to MMS, suggesting a damage-dependent functional link between the two proteins.

In order to clarify whether H2A.Z is involved in DNA damage responses and whether this involvement is Swr1 independent in *S. pombe*, we studied genetic interaction between two proteins before and after induction of DNA damage. Using spotting assay, we found that *pht1*Δ cells are sensitive to high dose of MMS (0.006%) and reintroducing of a wild type copy of *pht1* gene rescued the sensitive phenotype. However cells lacking *swr1* showed a slight sensitivity to MMS compared to *pht1*Δ cells. The double *swr1Δpht1Δ* mutant did not show a strong sensitivity to MMS as well, suggesting a weak correlation between two proteins after DNA insults. Additionally we monitored the size of yeast colonies in treated and untreated conditions. In basal condition deletion of *swr1 in pht1Δ* cells suppressed the slow growth of *pht1Δ* cells however after exposure to MMS this suppression was reduced as expected from our Podbat data.

In this study we proposed that H2A.Z is involved in DNA damage responses independent of Swr1 complex in *S. pombe*. However more studies are required to better understand the relationship between H2A.Z and Swr1 after DNA damage as it was beyond the scope of this paper. A cellular response to genotoxic insults is initiated by rapid transcription of genes involved in DNA repair. It has already been shown that H2A.Z is involved in transcriptional elongation by facilitating RNAPII progression. Moreover a recent study showed that the levels of H2A.Z enrichment at gene body region generally correlates with gene responsiveness and the lack of H2A.Z causes misregulation of genes that respond to a variety of stimuli (Coleman-Derr & Zilberman, 2012). I speculate that presence of H2A.Z at gene body region of DNA damage response genes could be a way to promote rapid transcription of genes involved in DNA damage repair.

3.2 PAPER II

Earlier it was shown that RNF20 knockdown human cell lines are resistant to 4-NQO (Svensson et al, 2012), indicating that H2Bub1 plays a role in DNA damage response. Deficient DNA damage responses results in chromosomal aberrations and genomic instability. In this study we aimed to elucidate the role of H2Bub1 in DNA damage response. We found that *brl1*Δ cells are resistant to 4-NQO in *S. pombe* similar to RNF20 knockdown human cell line. Next, cell cycle progression was monitored in WT and *htb1*-K119R (H2Bub1 deficient cells) cells using flow cytometry analysis before and after induction of DNA damage. This analysis showed that the cell cycle in *htb1*-K119R cells was not arrested after DNA insults. Adam G.West ‘s laboratory showed that the deletion of RNF20 led to spreading of heterochromatin and silencing of neighboring genes (Ma et al, 2011). We hypothesized that in H2Bub1 deficient cell, heterochromatin spread over genes involved in DNA damage response and caused defect in the repair processing pathway. To test this hypothesis we aimed to understand the role of H2Bub1 in the maintenance of epigenetic stability in *S. pombe*. We mapped the genome-wide distribution of heterochromatin mark H3K9me2 in WT and *htb1*-K119R cells in *S. pombe* using ChIP-chip. We found that genome-wide pattern of H3K9me2 in *htb1*-K119R cells was similar to WT with correlation coefficient of 0.83 except a region, which annotated to the centromeres. H3K9me2 was present at the central core domain of all three centromeres in *S. pombe*. Elevated levels of H3 and reduced levels of CENP-A^{Cnp1} at the central core domain of *htb1*-K119R cells was observed with respect to WT, suggesting a role for H2Bub1 in replacing the centromeric histone H3 with CENP-A^{Cnp1}. A previous study (Ma et al, 2011) proposed a role for H2Bub1

in the maintenance of chromatin boundary integrity. To examine the integrity of the chromatin barrier between pericentric heterochromatin and centromeric chromatin, we determined the levels of Sfc6, a component of TFIIIC, present at tRNA boundaries in WT and *htb1*-K119R cells. We observed that centromeric boundaries are not affected in H2Bub1 deficient cells because Sfc6 displayed similar levels in WT and *htb1*-K119R cells. We concluded that loss of H2Bub1 promotes *de novo* heterochromatin assembly at the central core domain of centromere.

To explore the role of H2Bub1 at centromeric chromatin, we further checked the H2Bub1 levels at the centromere of asynchronized cells. Even though it has been shown that histone H2B is present at the centromere H2Bub1 levels were not detectable at the centromere of asynchronized cells, suggesting that H2B might be ubiquitinated during a narrow window of the cell cycle. To examine H2Bub1 levels at the centromere at different stages of the cell cycle, we used *cdc25-22* temperature sensitive mutant to synchronize cells. We found a significant level of H2Bub1 at the centromere in G2-M phase. H2Bub1 is tightly coupled with RNAPII dependent transcription. In *S. pombe* H2Bub1 facilitates phosphorylation of RNAPII CTD to promote transcription. To find out whether H2Bub1 is linked to the transcription of the central core domain, we examined the levels of Ser5 phosphorylated RNAPII at different stages of the cell cycle at the centromere. Consistent with increased H2Bub1 levels, RNAPII Ser5-P levels elevated at G2-M phase, indicating that H2Bub1 mediates Ser5-P and general transcription of the centromere. Moreover, in *htb1*-K119R cells RNAPII levels at the central core domain was reduced compared to WT demonstrating H2Bub1 dependent recruitment of RNAPII to the centromeric chromatin.

Then we checked transcription levels at the centromeric chromatin both by using marker gene integrated at the central core domain and endogenous centromere transcripts. Centromeric DNA is transcribed and the transcripts are rapidly cleaved and degraded (Choi et al, 2011). To detect endogenous transcripts, we used *pfs2-11* temperature sensitive mutants where mRNA cleavage is compromised allowing the accumulation of RNA from the central core domain. We showed a reduction of transcripts from both marker gene and endogenous sequences in *htb1*-K119R cells, suggesting that H2Bub1 is required for RNAPII dependent transcription of the central core domain.

In order to understand whether H2Bub1 at the central core domain has any functional role, we exposed *htb1*-K119R cells to TBZ, which is a microtubule-destabilizing drug and found

that *htb1*-K119R cells are sensitive to TBZ, indicating that mutants possess defects in chromosome segregation. Moreover, by immunofluorecent staining, we showed that H2Bub1 deficient mutants exhibit defective chromosomal segregation because *htb1*-K119R cells frequently showed lagging and stretched chromosome.

Since H2B is monoubiquitinated in a narrow window of the cell cycle and is removed immediately after G2-M phase, we asked whether removal of H2Bub1 is via nucleosome eviction or deubiquitination. To answer this question first, we checked nucleosomal turnover in centromeric central core domain using epitope-tagged H3 under an invertase-inducible promoter. We measured the levels of newly produced H3 containing nucleosome, which has been incorporated in the central core domain after sucrose induction. As a result, the level of H3 containing nucleosomes has been reduced at the central core domain of centromere in *htb1*-K119R cells compared to WT. However, it has already been shown that deposition of histone H3 at heterochromatic regions is lower than euchromatic regions and in *htb1*-K119R cells central core domain is enriched with heterochromatin mark H3K9me2. We argue that histone H3 is required for heterochromatin formation and lower H3 eviction could cause the accumulation of H3 and eventually modifying to H3K9me2. However, to measure the precise nucleosome turnover rate at the central core domain, we need to use more efficient method such as RITE system. In addition, Ubp8 and Ubp16, H2Bub1 deubiquitinases, single and double deleted mutants were not sensitive to TBZ suggesting that H2B deubiquitination is dispensable for centromeric chromatin assembly.

A functional centromere is prerequired for the proper and stable formation of the kinetochore, which is a protein complex that assembles onto centromeric loci. The kinetochore is involved in the attachment of chromosomes to microtubules and contributes to proper chromosome segregation. Since H2Bub1 regulates gene expression, we asked whether loss of H2Bub1 could potentially affect transcription level of kinetochore proteins leading to aberrant kinetochore structure. To explore this in more detail, we used microarray analysis to study global changes in gene transcription levels in *htb1*-K119R cells compared to WT. Our analysis indicated that loss of H2Bub1 did not affect transcription levels of kinetochore proteins in *S. pombe*. However aberrant centromere structure in *htb1*-K119R cells could lead to mislocalization of kinetochore proteins, which eventually cause chromosome missegregation.

Moreover, we checked whether Brl1 is involved in H2B ubiquitination at the central core domain of the centromere in *S. pombe*. We found that the H2Bub1 level was significantly reduced upon deletion of *brl1*. We found that Brl1 is present at the central core domain during G2-M phase because deletion of *brl1* led to heterochromatin assembly at central core domain, which caused TBZ sensitivity.

To test whether the role of H2Bub1 at centromeric chromatin is conserved in human cells, first, we determined whether RNF20 is recruited to CENP-A containing nucleosomes in human cells using *in situ* proximity ligation assay. We found that RNF20 and RNAPII are present at CENP-A containing chromatin in human cells. Then, we checked CENP-A levels at ALR repeats, centromeric satellite repeats (Murphy & Karpen, 1998), in RNF20 depleted cells and found that CENP-A levels have been reduced compared to the control. Moreover, we checked whether knockdown of RNF20 in a human cell line would lead to segregation defects. To answer this question, we measured micronuclei in control and RNF20 depleted cells and found that RNF20 knockdown led to chromosome loss. However, H2Bub1 is involved in homologous recombination (HR) (Chernikova et al, 2012). We checked whether chromosome loss in RNF20 knockdown cells is the consequence of unrepaired DNA damage or aberrant segregation by staining with γH2AX foci. We found that RNF20 knockdown led to double strand breaks (DSB) but also increased chromosome loss unrelated to DNA damage.

In summary, we showed that defect in H2Bub1 alters the centromeric chromatin structure leading to heterochromatin formation in the central core domain. Heterochromatization results in the reduced levels of centromeric transcription and abnormal chromosome segregation. Cell cycle dependent H2Bub1 is required for centromeric transcription, which results in a functional centromere (Figure 7).

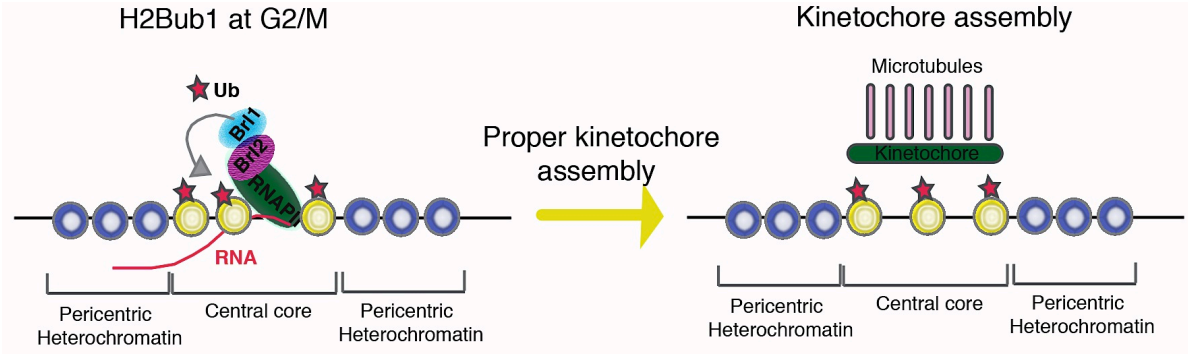

Figure 7. H2Bub1 at the central core domain of centromere. Histone H2B is monoubiquitinated at the central core domain of centromere during G2-M phase of the cell cycle. H2Bub1 mediated by E3 ligases Brl1/Brl2 is required for RNAPII dependent transcription of the central core domain, which is involved in the formation of centromeric specific chromatin structure and kinetochore assembly. H2Bub1 is essential for proper kinetochore assembly and accurate chromosome segregation during mitosis.

3.3 PAPER III

Previous studies have shown that Epe1 is localized at *IRC* boundaries surrounding cen1 and cen3. These boundaries are essential for preventing pericentric heterochromatin spread into neighboring regions (Zofall & Grewal, 2006). However, we still know very little about proteins are involved in boundary function. In this study we aimed to characterize the proteins that are involved in the maintenance of heterochromatin boundaries in *S. pombe*. To this purpose, we generated a random mutant library using the *Hermes* transposon mutagenesis system. The silencing of two reporter genes *ade6+* and *ura4+* inserted into the euchromatic region adjacent to the heterochromatin at the mating type locus was monitored by spotting assays. In fission yeast the mating-type region consist of transcriptionally repressed region (K region) and active euchromatin (L region), which are separated by inverted repeats *IRs*. Reporter gene *ade6+* was inserted at the Inverted Repeat Left (*IR-L*) whereas *ura4+* integrated 1.2 Kb further into the L region. We screened for mutant that exhibited red color on low adenine plates and grew on plates containing 5-FOA (5-Flouroorotic acid) by silencing of integrated reporter genes. We identified Leo1 (SPBC13E7.08c), a component of the Paf1C, required for preventing heterochromatin spread into euchromatin at the mating type locus. In parallel H3K9me2 levels at this locus were examined using ChIP-qPCR. We found that in the absence of Leo1 and Paf1 H3K9me2 levels were significantly increased at the mating type locus. Paf1 complex is composed of the subunits Paf1, Leo1, Tpr1 and Cdc73 in *S. pombe*. We examined whether deletion of the other subunits of Paf1 complex Tpr1 and Cdc73 exhibit the same phenotype as *leo1Δ and*

*paf1*Δ cells. However, *tpr1* and *cdc73* deleted cells did not show the spread of heterochromatin into adjacent euchromatic region at the mating type locus indicating that only Paf1/Leo1 are involved in preventing of heterochromatin propagation. Then we asked whether the heterochromatin propagation seen in *leo1*Δ cells could be stably inherited via mitosis as it has been shown for *epe1*Δ cells. To answer this question we selected colonies from 5-FOA and –Ura plates and after growing them on non-selective media, they were restricted on selective plates. Cells that inherited silencing of *ura4*+ grew well on the 5-FOA containing plates. Indeed this observation indicated that deletion of Paf1/Leo1 led to heterochromatin stabilization, which is inherited during mitosis.

Paf1 complex is involved in RNAPII dependent transcription. Paf1C participates in the recruitment of enzymes that catalyze H2Bub1 and H3K4me2. We already showed that deficiency in H2Bub1 leads to *de novo* heterochromatin assembly. To elucidate if heterochromatin assembly in the absence of Paf1/Leo1 is caused by impaired H2Bub1 levels, first we checked H2Bub1 levels in *leo1*Δ cells and we found that H2Bub1 levels were reduced in *leo1* deleted cells compared to WT. Next, we examined heterochromatin propagation across *IR* boundary in mutants deficient in H2Bub1 and H3K4me2. Our results indicated that the role of Leo1 in heterochromatin formation is separated from its role in recruiting H2Bub1 and H3K4me2.

Our previous experiments showed that Paf1/Leo1 behave like anti silencing factor Epe1, as deletion of Paf1/Leo1 led to heterochromatin spread at the mating type locus and this heterochromatin formation was stably maintained. We decided to check whether there is any genetic interaction between Paf1/Leo1 and Epe1. Epe1 is degraded by Cul4-Ddb1 ubiquitin ligase and deletion of Ddb1 causes accumulation of Epe1 at heterochromatin domains. Cells lacking Ddb1 show a silencing defect at heterochromatic regions. To determine genetic interaction between Epe1 and Paf1/Leo1, we examined whether deletion of Paf1/Leo1 in Ddb1 deleted cells can rescue heterochromatic silencing defect at the silenced K region of the mating type locus seen in *ddb1*Δ cell. We found that Paf1/Leo1 deletion rescued silencing defect suggesting a genetic interaction between two proteins.

To clarify the role of Leo1 in heterochromatin formation, we performed Chip-exo for high-resolution genome-wide mapping H3K9me2 in *leo1*Δ cells. We found that in *leo1*Δ cells, H3K9me2 levels at pericentric regions were unchanged however; there were increased levels of H3K9me2 at the mating type locus and facultative heterochromatin islands over meiotic

genes and retrotransposons (Tf2s). The genome of the laboratory strain of fission yeast contains 13 retrotransposable elements Tf2s that are flanked by LTRs. The Tf2s are transcribed but transcripts are degraded by the exosome. To confirm heterochromatin stabilization we checked the levels of $HP1^{Swi6}$ at the mating type locus and Tf2s in *leo1*Δ cells using ChIP-qPCR. Similar to H3K9me2, $HP1^{Swi6}$ levels were increased at indicated regions confirming heterochromatin stabilization in the absence of Leo1.

It has been shown that Leo1 binds to transcribed RNA and is involved in the recruitment of m-RNA 3′end processing factors (Dermody & Buratowski, 2010). Transcription termination defect leads to siRNA dependent *de novo* heterochromatin formation (Kowalik et al, 2015). We wanted to assess whether deletion of Leo1 disrupts 3′end processing and termination leading to the accumulation of aberrant transcripts and heterochromatin assembly. For this purpose, we checked whether deletion of Res2 and Ctf2, factors involved in RNA termination, leads to heterochromatin spread across *IR* boundary at the mating type locus as we have seen in Paf1/Leo1 deleted cells. Our spotting assay using marker genes, showed that the deletion of Res2 or Ctf1 did not cause silencing of marker genes indicating that impaired termination of transcription do not lead to heterochromatin spread at the mating type locus. Moreover, we checked whether deletion of Res2 or Ctf1 can rescue heterochromatin silencing defect at K region in *ddb1*Δ cells as we have observed in Paf1/Leo1 deleted cells. We found that in contrast to Paf1/Leo1 deleted cell, deletion of Res2 or Ctf1 did not restore silencing at K region in *ddb1*Δ cells suggesting that there is no functional association between Res2/Ctf1 and Leo1 or Epe1. This observation indicated that impaired termination of transcription is not able to stabilize heterochromatin in Leo1 and Paf1 deleted cells. Proper transcription termination inhibits *de novo* heterochromatin assembly however defective transcription termination is not the major cause of heterochromatin formation in *leo1*Δ and *paf1*Δ cells.

An earlier study showed that the deletion of Rrp6 involved in mRNA degradation leads to RNAi dependent heterochromatin assembly over meiotic genes and Tf2s in *S. pombe*. We hypothesized that similarly in *leo1*Δ cells aberrant transcripts instead of exosome degradation are loaded into the RNAi machinery, which leads to siRNA generation and heterochromatin assembly. To check our hypothesis, we performed sRNA (small RNA) sequencing which includes siRNA in WT and *leo1*Δ cells and we used *ago1*Δ and *rrp6*Δ cells as control to study sRNA population. It has been shown that in Ago1 deleted cells the pericentric siRNA population was reduced whereas in Rrp6 deleted cells the siRNA population from Tf2s was

mainly increased versus WT. By mapping sRNA to the genome we found that the sRNA population homologous to the pericentric region in *leo1*Δ cells was reduced compared to WT. However, the sRNA population homologous to Tf2s in WT and *leo1*Δ cells was sense direction, which was different from double stranded sRNA population in *rrp6*Δ cells. This indicates that facultative heterochromatin formation over Tf2s in Leo1 deleted cells is RNAi independent. In addition, we observed the reduced levels of sRNA population over *IRC* boundary elements in *leo1*Δ cells. Our results indicated that reduced levels of siRNA at pericentric region in *leo1*Δ cells is sufficient to establish and maintain the H3K9me2 levels because we did not observed the reduced levels of H3K9me2 in *leo1*Δ cells at pericentric region.

The correlation between nucleosome turnover and the epigenetic stability of heterochromatin and the role of Leo1 in destabilizing heterochromatin encouraged us to check histone turnover in *leo1*Δ cells. For this purpose, we used the RITE system. In this system epitope tag of histone H3 **(***hht2*+**)** swap from HA (old nucleosome) to T7 (new nucleosome) using Cre/*Loxp.* We induced the switch and after 2 hours, we collected samples for ChIP-qPCR. Our experiment showed the reduced level of newly incorporated H3 histones in *leo1*Δ cells at Tf2s, mating type locus and pericentric region. However, to conclude that Paf1/Leo1 is involved in histone turnover as a general mechanism, we checked several euchromatic loci including *IRC* boundary elements and found that RNAPII transcribed loci are associated with lower histone turnover in *leo1*Δ cell compared to WT. Histone turnover rate at RNAPIII transcribed region including tRNA gene was not affected by loss of Leo1. Moreover, we checked whether the deletion of Paf1/Leo1 can rescue silencing defect in Pob3 deleted cells (FACT subunit) and we found that the deletion of Paf1/Leo1 is not able to rescued effect of *pob3*Δ suggesting that these two proteins act synergistically.

To clarify whether reduced histone turnover in *leo1*Δ cells is a cause or consequence of heterochromatin stabilization, we asked whether the deletion of Mst2 can rescue silencing defect at K region in *ddb1*Δ cells similar to Paf1/Leo1 deleted cells. Mst2 is a histone acetyltransferase, which is involved in H3K14 methylation and regulation of histone turnover rate at heterochromatic regions. Our experiment showed that similar to *leo1*Δ cell loss of Mst2 can restore silencing in *ddb1*Δ cells by affecting histone turnover. This indicated that Leo1 regulates histone turnover and heterochromatin stabilization in *leo1*Δ cells is the consequence of reduced histone turnover. In summary our data revealed that Leo1-Paf1 is

involved in the maintenance of euchromatic regions by promoting histone turnover through RNAPII dependent transcription (Figure 8).

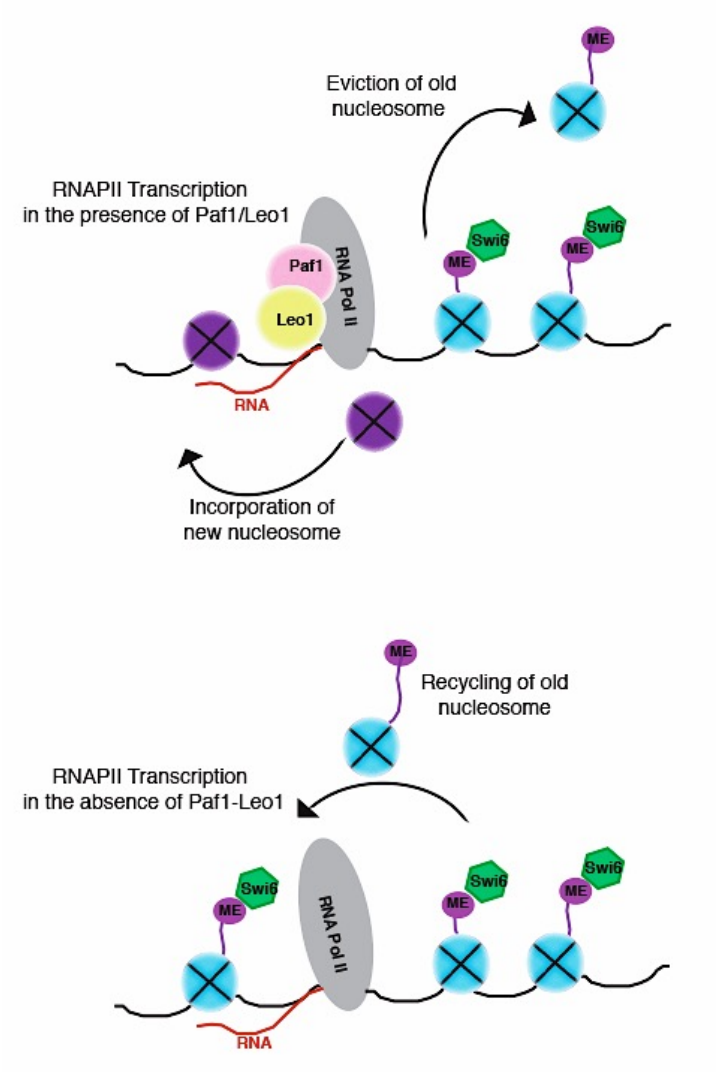

Figure 8. Role of Paf1/Leo1 in nucleosome turnover. During RNAPII dependent transcription PAF1/Leo1 mediates eviction of old histones with specific modifications and by that they prevent heterochromatin stabilization. In the absence of Paf1/Leo1 old histones fail to evict which leads to heterochromatin stabilization.

4 FINAL CONCLUSIONS AND PERSPECTIVES

The aim of this thesis was to understand how transcription of chromatin and histone variants assembly modulates chromatin structure. In this thesis we explored how transcription of non-coding regions on the genome could influence the structure of chromatin domains. We showed that transcription dependent histone turnover promotes eviction of old histones and by that prevents heterochromatin from either *de novo* formation or spread into neighboring region. In **Paper II**, We showed that H2Bub1 dependent transcription of central core domain of centromere is required for the replacement of histone H3 with CENP-A^{Cnp1} and prevention of *de novo* heterochromatin formation. Moreover, we showed that H2Bub1 is required for proper chromosomal segregation during mitosis. In **paper III,** we showed that Paf1/Leo1 is required for the maintenance of euchromatin by promoting histone turnover. Loss of Leo1 conserves parental histone modifications, which cause heterochromatin stabilization. In **paper I,** we suggest a role for H2A.Z in DNA damage responses that is independent of Swr1. We proposed that H2A.Z is present at gene body region of stress response genes in a Swr1 independent fashion.

4.1 TRANSCRIPTION AND CHROMATIN STRUCTURE

In this thesis we showed for the first time that H2B is monoubiquitinated at the central core domain in a cell cycle dependent manner. We showed that H2Bub1 is required for the transcription of central core domain and proper kinetochore assembly resulting in accurate chromosomal segregation. We showed that the role of H2Bub1 at CENP-A containing chromatin is conserved in human cells as well. Aneuploidy results from missegregation of chromosomes and is observed in many cancers. Moreover, RNF20 and consequently H2Bub1 levels are often reduced in cancer cells, which indicate that H2Bub1 plays an essential role in the maintenance of genomic stability through establishing proper chromatin structure of centromere and promoting accurate chromosomal segregation.

In the absence of H2Bub1 heterochromatin mark H3K9me2 is enriched at centromeric chromatin. This was not clear whether RNAi machinery directs H3K9me2 assembly at the central core domain in H2Bub1 deficient cells. Later we performed small RNA sequencing and showed that siRNA is absent from the central core domain of *htb1*-K119R cells indicating that heterochromatin assembly is RNAi independent. Thus, there is a possibility for H2Bub1 in regulating H3 eviction from central core domain and preventing heterochromatin assembly. To further study the mechanism of heterochromatin assembly the

RITE system can be used to study histone turnover rate at the central core domain of *htb1*-K119R cells. Although we examined histone turnover rate using epitope-tagged H3 under an invertase-inducible promoter but this system is very leaky and has limitations. They can yield undesirable levels of overexpression, which can cause nonphysiological consequences.

The human Paf1C (hPaf1C) is involved in both transcription initiation and elongation. In addition to transcription, Paf1C is associated with the maintenance of ESC (Embryonic stem cell) identity in mammals (Ding et al, 2009). The hPaf1 is overexpressed in the pancreatic cancer cell line, suggesting that the hPaf1 gene plays a role as an oncogene (Moniaux et al, 2006). Moreover, the Paf1C has been implicated in Wnt signaling pathway, which indicates that this protein complex participates in biological processes such as embryonic development (Mosimann et al, 2006). Recently it has been shown that Leo1 subunit of the Paf1C is involved in the maintenance of *IRC* boundaries via recruitment of H4K16 acetylation in *S. pombe* (Verrier et al, 2015). In this thesis we introduce a new role for Paf1C, we showed that Paf1/Leo1 regulates transcription dependent histones turnover, which is necessary for maintenance of euchromatin. We observed that Paf1/Leo1 similar to Mst2 prevents heterochromatin spread across *IR* boundary at the mating type locus. Mst2 acts on the chromatin-bound histones. This suggests that Paf1/Leo1 destabilizes nucleosomes and promotes eviction of old histones. In the absence of Paf1/Leo1 heterodimer preservation of old histones results in heterochromatin stabilization that can be inherited for several cell division. However the mechanism by which Leo1 promotes histone turnover is not clear. It has been shown that in human cells N-terminal region of Leo1 has H3 binding affinity, which is conserved between human and *S. pombe.* Further studies are required to understand whether Paf1/Leo1 acts as a chaperon to interact with histone H3.

H2Bub1 is partially dependent on Paf1/Leo1 because the deletion of Leo1 reduced global H2Bub1 levels. We examined H3K9me2 levels at the central core domain of centromere in *leo1*Δ cells. However, H3K9me2 was not present at the central core domain in *leo1*Δ cells suggesting that Paf1C is not involved in the transcription of the central core domain. Moreover, we showed that Paf1/Leo1 dependent histone turnover is necessary for maintenance of *IR* boundaries between heterochromatin and euchromatin. Loss of Paf1/Leo1 led to heterochromatin spreading across *IR* boundaries, which is not affected in H2Bub1 deficient cells. This observation suggests that H2Bub1 is not required for the targeting of histone turnover at boundary regions as we have seen in Paf1/Leo1.

However, loss of H2Bub1 and Paf1/Leo1 heterodimer resulted in a reduced level of siRNA population mapped to pericentric region. I speculate that both H2Bub1 and Paf1/Leo1 are involved in transcription of pericentric region, which is RNAPII dependent. H2Bub1 and Paf1/Leo1 are involved in transcription elongation and loss of H2Bub1 and Paf1C may result in reduction of pericentric transcripts. However more studies are required to elucidate the role of H2Bub1 and Paf1C in the transcription of pericentric DNA. Nevertheless the loss of H2Bub1 and Paf1/Leo1 led to RNAi independent heterochromatin assembly by interplay between histone stability and transcription.

4.2 H2BUB1 AND DNA DAMAGE

Apart form its role in transcription; H2Bub1 has a role in DNA damage responses. It has been shown that RNF20/RNF40 are present at the site of DSBs when general transcription is shut off using Actinomycin D (Moyal et al, 2011). DSBs arise from multiple sources including arrest of the replication machinery and in RNF20 knockdown cells these lesions are accumulated which leads to chromosomal instability. It is not clear how H2Bub1 is involved in DNA damage responses but by promoting centromeric transcription and accurate chromosomal segregation H2Bub1 prevents DSB formation and protects genomic stability. In RNF20 depleted cells unrepaired chromosomes breaks and chromosomal segregation defects act together to accumulate chromosomal aberrations.

H2A.Z is involved in the regulation of the DNA damage response however; further studies are required to understand the role of H2A.Z in DNA damage responses in *S. pombe*. The correlation between *pht1* and genes involved in DNA damage responses can be assessed before and after DNA damage insults using epistasis analysis in *S. pombe.* In addition H2Bub1 is associated with H2A.Z. In *S. pombe* double deleted mutation of *pht1*Δ *htb1*-K119R is lethal. Lack of H2A.Z interferes with chromosome architecture and results in chromosome breakage and deficiency in H2Bub1 leads to abnormal centromere chromatin structure and segregation defect. Taken together I speculate that in double deleted mutant *pht1*Δ *htb1*-K119R cell are not able to divide properly. They might undergo massive chromosomal aberrations, which leads to cell death.

In conclusion, these studies expand our understanding of the interplay between transcription, histone turnover and histone variants in modulating chromatin.

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